different properties which has been named cephalosporin N. Cephalosporin P consists of acidic substances that are soluble in organic solvents, and are active mainly against Gram-positive organisms. Cephalosporin N cannot be extracted from aqueous solution by common organic solvents and is active against both Gram-positive and Gram-negative organisms.

2. Crude cephalosporin P can contain at least five antibiotics, which have been named cephalosporin P_1, P_2, P_3, P_4 and P_5. Cephalosporin P_1 was the major active substance in extracts of the culture fluids that were used in the present work. By counter-current distribution between solvents and chromatography on Florisil, cephalosporins P_1, P_2 and P_4 have been isolated in crystalline form.

3. Cephalosporin P_1 is almost twice as active as helvolic acid against *Staph. aureus*, but the two antibiotics resemble each other in certain chemical and biological properties. Staphylococci that acquire resistance to cephalosporin P_1 by growing in its presence become, at the same time, resistant to helvolic acid. Similarly, resistance acquired to helvolic acid is accompanied by an increase in resistance to the cephalosporin. Cephalosporin P_1 is inactivated by a substance that is present in a preparation of penicillinase. This substance has been provisionally named P-cephalosporinase. The same preparation of penicillinase also causes the inactivation of cephalosporins P_2, P_3, P_4, P_5, and helvolic acid.

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Tissue Fractionation Studies

1. THE EXISTENCE OF A MITOCHONDRIA-LINKED, ENZYMICALLY INACTIVE FORM OF ACID PHOSPHATASE IN RAT-LIVER TISSUE

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In the course of an investigation on the specific glucose-6-phosphatase of liver, the results of which have been published elsewhere (Duve, Berthet, Hers & Dupret, 1949; Hers, Berthet, Berthet & Duve, 1951), some peculiar observations were made on the unspecific acid phosphatase which is also present in hepatic tissue. Evidence was obtained, indicating that this enzyme is associated with mitochondrial granules by some kind of labile linkage, which renders it inactive towards added glycerophosphate at pH 5.

Experiments which substantiate this conclusion are reported in the present paper. A short account of these findings has already been published in preliminary form (Duve, Berthet, Berthet & Appelmans, 1951).

METHODS

Fractionation of tissue constituents. Rats, fasted for 12 hr. were killed by a blow on the head and bled, and the liver taken out immediately and immersed in an ice-cold isotonic solution. In most cases, the chilled organ was pressed through a wire mesh (Claude, 1946), in order to remove the main vascular and connective network, and the resulting pulp was weighed and homogenized in cold 0·25 M-sucrose, using a Pyrex glass homogenizer of the Potter-Elvehjem type (Potter & Elvehjem, 1936).

In the subsequent fractionation, the technique of Claude (1946), as modified by Schneider (1948), was followed in all

essential details. The homogenate was first centrifuged 10 min. at 600 *g* in the Potter-Elvehjem tube. After decanting the supernatant, the precipitate, containing, in addition to red blood-cells, unbroken liver cells and gross cytoplasmic debris, all the nuclei and part of the mitochondria, was rehomogenized with a new quantity of fluid and recentrifuged. This procedure was repeated a second time. The final precipitate, which contained no intact cells or coarse cellular debris and was free of most of the mitochondria, represented the nuclei fraction. The combined supernatants were used for the isolation of the other fractions.

To reconstitute the initial homogenate, samples of the combined supernatants and of the nuclear fraction, representing the same amount of original tissue, were mixed together, furnishing a more reliable preparation than the actual first homogenate, in which all the cells were not entirely broken up.

The mitochondria were separated from the supernatants of the first centrifugations by centrifuging 10 min. at 8500 *g*, using lucite tubes and an MSE 'Major' (Measuring and Scientific Equipment Ltd., London, S.W. 1) centrifuge with multispeed attachment. The supernatant was removed carefully with a medicine dropper. The precipitate was resuspended in a new quantity of fluid by means of a motor-driven glass pestle, whose extremity had been blown into a spherical bulb fitting closely into the bottom of the centrifuge tube. The mitochondria were washed either once or twice. When washed mitochondria were prepared, the procedure was stopped here.

In complete fractionations, the supernatant and washings of the preceding centrifugations were combined and centrifuged for 90 min. at 20,000 *g* to separate the microsomes. The precipitate was washed once or twice, using the same technique as that described for washing the mitochondria. By combining the supernatant and washings of these centrifugations, the final supernatant was obtained.

Samples of each fraction were kept for enzyme assays. In making up the volumes of the various fractions, care was taken to render them all comparable with each other and with the initial homogenate, in order to allow a quantitative study of the enzyme distribution to be made.

The entire fractionation was carried out in the cold room. In order to keep the temperature as low as possible during the high-speed centrifugations, the centrifuge was allowed to run with the lid open in a room at $-5°$. Despite this precaution, the temperature of the rotor reached 10° at the end of the long runs at 20,000 *g*. The gravitational field strengths given are for the middle of the centrifuge tubes.

Assay of enzyme activity. The enzymic tests were performed at pH 5 and 38°, using commercial β-glycerophosphate (Judex) as substrate. In most experiments, a universal phosphate-free buffer was used, containing equivalent amounts of sodium acetate, cacodylate and borate, and adjusted with HCl (*ABC* buffer; Duve *et al.* 1949). These buffer mixtures, which cover the pH range from 4 to 10, have been used in this laboratory to study a number of enzymes, including several phosphatases, phosphorylase, phosphoglucomutase, hexokinase and fructokinase. They exert few inhibitions and are easily prepared from stock solutions which keep indefinitely. Their use in this work, which started more or less fortuitously, was continued as a matter of routine after it was found that acid phosphatase was not inhibited.

The tests were made by mixing equal volumes of the enzyme preparation, in 0·25 M-sucrose, and of the substrate which contained 0·16 M-*ABC* buffer and 0·1 M-sodium glycerophosphate adjusted to pH 5. The incubation time varied between 10 and 20 min. The reaction was stopped with 8% (w/v) trichloroacetic acid and the inorganic P determined on the filtrate according to Fiske & Subbarow (1925), using a Klett-Summerson photoelectric colorimeter. Suitable blanks were always run.

Preliminary assays performed with purified solutions of acid phosphatase showed that the reaction proceeded linearly with time, and that the activity was proportional to the amount of enzyme added, under the conditions of the tests.

RESULTS

Preliminary observations. The main observations which led to the present investigation are recorded in Tables 1 and 2. In this experiment, a complete fractionation of liver tissue was performed according to the technique outlined above, and tests for acid phosphatase carried out on the various fractions. The results of these tests (Table 1) show that a very poor activity balance sheet was obtained. Moreover, the activity of the initial homogenate was low, about one-tenth of the value usually observed in earlier experiments on aqueous liver extracts prepared in the Waring blender (Duve *et al.* 1949).

A second series of tests, made 5 days later on the fractions which had been kept at 2° showed that considerable activation of the preparations had occurred (Table 1). The aged homogenate exhibited a fairly 'normal' value, the recovery was more satisfactory and more than 50% of the total enzyme activity was found in the mitochondria fraction.

Table 1. *Cellular distribution of acid phosphatase activity in fresh and aged preparations*

(Fractionation of a 0·25 M-sucrose homogenate of rat liver.)

Fraction	Acid phosphatase activity* (mg. P/g. original tissue/10 min.) Fresh preparations	After 5 days at 2°
Initial homogenate	0·16 (100)	1·34 (100)
Nuclei, washed twice	0·04 (25)	0·16 (12)
Mitochondria, washed twice	0·10 (63)	0·70 (52)
Microsomes, washed once	0·09 (56)	0·15 (11)
Final supernatant	0·09 (56)	0·14 (10)
Recovery	0·32 (200)	1·15 (85)

* Figures in brackets show activity distribution as percentage.

The same mitochondria were kept for 13 days at 2°, and then separated by centrifugation from the suspending fluid and washed once. Activity tests showed that only a small proportion of the enzyme had remained attached to the granules in the activated preparation (Table 2).

Two facts emerge from these experiments. In a fresh homogenate prepared in isotonic sucrose by the Potter-Elvehjem method, the acid phosphatase activity is low and is associated to a large extent with the mitochondria fraction; in an aged homogenate the activity is high and is no longer associated with the mitochondria.

Table 2. *Liberation of acid phosphatase from aged mitochondria*

(Mitochondria of Table 1, after 13 days at 2°, centrifuged 10 min. at 8500 *g* and washed once with 0·25 M-sucrose.)

Fraction	Acid phosphatase activity* (mg. P/g. original tissue/10 min.)
Aged mitochondria	0·73 (100)
Supernatant of first centrifugation	0·62 (85)
Precipitate of first centrifugation	0·11 (15)
Supernatant of second centrifugation (washing)	0·08 (11)
Precipitate of second centrifugation (washed)	0·03 (4)

* Figures in brackets show activity distribution as percentage.

It seemed likely that the low activity of the fresh preparation was a direct consequence of the bound state of the enzyme, and that 'activation' and 'liberation' were two related aspects of the same phenomenon. This conclusion has been substantiated by a large number of experiments, of which only representative examples will be described below.

Table 3. *Acid phosphatase activity after homogenization in the Waring blender*

(0·25 M-Sucrose homogenate of rat liver, prepared in the Waring blender (2 min.), centrifuged for 10 min. at 8500 *g*.)

Fraction	Acid phosphatase activity (mg. P/g. original tissue/10 min.)	
	Fresh preparations	After 2 days at 2°
Initial homogenate	1·45	1·70
Supernatant from centrifugation	1·50	1·60

Effect of the homogenizing device. When the Waring blender is used to homogenize the tissue, preparations of high activity are obtained, even in 0·25 M-sucrose or other isotonic solutions. In these preparations, the activity is present to a major extent in the supernatant after 10 min. centrifugation at 8500 *g*, and increases only slightly with ageing (Table 3). Experiments will be described below, showing that washed mitochondria exposed for 3 min. to the action of the Waring blender release their entire content of acid phosphatase.

These results explain the high activities observed earlier in water extracts prepared in the Waring blender (Duve *et al.* 1949). In these experiments, a second factor helped to release the enzyme from the mitochondrial complex, namely the distilled water used as homogenizing medium (see below).

The use of the Potter-Elvehjem homogenizer decreases to a large extent, but does not abolish completely the mechanical damage to the complex. The results of Table 4 indicate that a certain amount of enzyme, varying between 3 and 10 % of the total content of the mitochondria, is brought into solution with each homogenization. The quantities actually released are probably greater than those given in Table 4, since mitochondria adsorb a fair proportion of the liberated enzyme (Berthet, Berthet, Applemans & Duve, 1951).

Table 4. *Liberation of acid phosphatase by the Potter-Elvehjem homogenizer*

(Suspension of washed mitochondria submitted to three successive homogenizations, each of 2 min. duration. Before the experiment and after each run, a sample of the suspension is taken and centrifuged 10 min. at 20,000 *g*. Enzyme tests performed on the supernatants. The total activity, determined after exposure to the Waring blender, was 0·93 mg. P/g. original tissue/10 min.)

Number of homogenizations	Soluble acid phosphatase activity	
	mg. P/g. original tissue/10 min.	Percentage of total
0	0·038	4
1	0·063	7
2	0·10	11
3	0·20	21·5

Even the procedure used to resuspend the mitochondria after centrifugation must release a small quantity of enzyme, since suspensions of washed mitochondria always contain a little soluble activity.

Effect of the homogenizing medium. Various tests have been performed in order to find out in which homogenizing medium the highest proportion of bound enzyme can be recovered. Twin experiments were usually carried out, in which samples of the same liver pulp were homogenized in two different media. The two homogenates were then tested for their acid phosphatase activity, the lowest activity being taken as evidence that more of the enzyme was mitochondria linked. In some cases, the stability of the enzyme-mitochondria complex was also studied.

The following media were investigated: distilled water, 0·25 M-sucrose, 0·88 M-sucrose, 0·25 M-sucrose +7 % (w/v) serum albumin, 0·15 and 0·45 M-potassium chloride. Except for distilled water which causes a rapid liberation of the enzyme, all the above media furnish preparations of approximately

the same low activity. However, the data represented graphically in Fig. 1 show that spontaneous activation occurs more rapidly in isotonic potassium chloride than in isotonic sucrose.

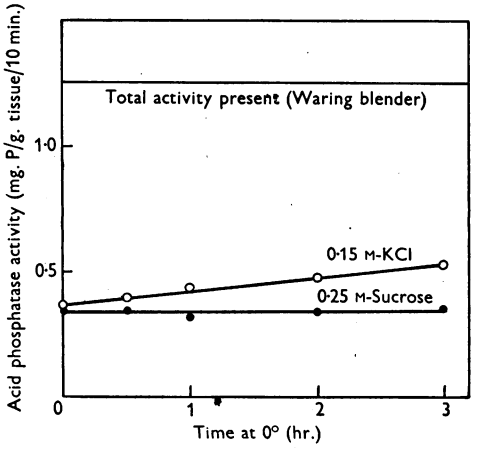

Fig. 1. Influence of KCl on spontaneous activation. Rat-liver pulp divided in two parts. Part 1 homogenized with 0·25M-sucrose; part 2 with 0·15M-KCl. The two homogenates kept at 0° and assayed regularly for acid phosphatase activity.

The best medium to obtain a high yield of stable mitochondria-linked acid phosphatase from rat liver appears to be salt free 0·25M-sucrose. The 0·88M-sucrose, advocated by Hogeboom, Schneider & Pallade (1948) for the preparation of intact mitochondria, has not proved more effective. Hogeboom & Schneider (1950) found that a change of 0·88–0·25M-sucrose does not alter the distribution of total nitrogen, cytochrome *c* and a number of enzyme systems, and now recommend the latter medium for practical reasons.

Differential measurement of free and bound activity. The quantitative measurement of the total acid phosphatase content of a preparation offers no special difficulty, since a short treatment in the Waring blender produces complete activation. The data of Table 5 indicate that alternate freezing and thawing, if repeated at least six times, may be used for the same purpose.

On the other hand, in determining the free activity of preparations containing the enzyme partly in the bound form, precautions have to be taken against activation during assay. Various experiments were performed in order to assess the amount of activation occurring under different conditions.

In Fig. 2 are recorded the results of an experiment, in which washed intact mitochondria (*I*) and the same mitochondria, fully activated by a short preliminary exposure to the Waring blender (*B*), were incubated under two different conditions:

Table 5. *Activation by freezing and thawing*

(Preparations 1 and 3: mitochondria isolated from a 0·25M-sucrose homogenate of rat liver, washed once. Preparation 2: precipitate after 1 hr. centrifugation at 20,000*g* of a 0·25M-sucrose homogenate of rat liver, containing nuclei, mitochondria and most of the microsomes, unwashed.)

Preparation no.	No. of successive freezings and thawings	Acid phosphatase activity (mg. P/g. original tissue/10 min.)
1	0	0·46
	1	0·70
	2	1·09
	3	1·18
2	0	0·16
	1	0·59
	2	0·78
	3	0·83
	4	0·82
	5	0·89
3	0	0·36
	6	1·15
	9	1·16
	12	1·20
	15	1·18
	16	1·19
Preparation 3 after 3 min. exposure to the Waring blender	0	1·16

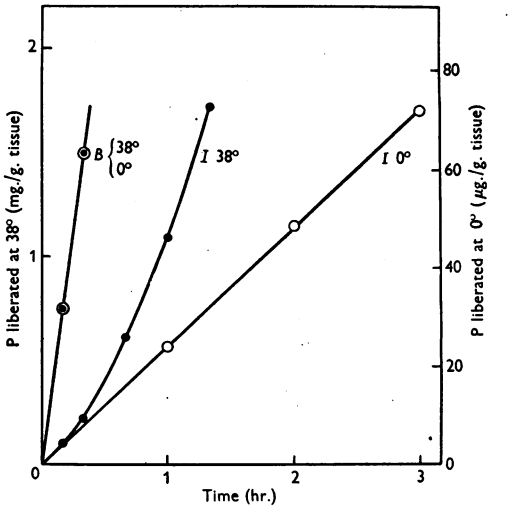

Fig. 2. Activation during the enzymic tests. *I*, washed mitochondria, intact; *B*, same preparation, treated 3 min. in the Waring blender. Incubation conditions: (*a*) 38°, pH 5, equal vol. of suspension in 0·25M-sucrose and 0·16M-*ABC* buffer + 0·1M-glycerophosphate. (*b*) 0°, pH 6·1, equal vol. of suspension in 0·25M-sucrose and 0·25M-sucrose + 0·1M-glycerophosphate.

(1) at 38° under the standard assay conditions; (2) at 0°, in 0·25M-sucrose containing 0·05M-glycerophosphate adjusted to pH 6·1. The course of liberation of inorganic P with time was followed.

As expected, the liberation of inorganic P is a linear function of time, both at 0° and at 38°, with the blender-treated preparation. It is 23·6 times slower at 0° than at 38° and the two curves may be made to coincide by using two different ordinate units bearing this ratio to each other. Plotted in the same manner, the curves for intact mitochondria coincide for the first 10 min., but subsequently diverge. The curve at 0° remains linear, indicating that no activation occurs in the test at low temperature. On the contrary, at 38°, progressive activation takes place as shown by the increasing rate of liberation of inorganic P.

The most important finding of this experiment is that the activation which occurs under the standard assay conditions does not begin immediately, but has a certain lag period. This lag period has been observed regularly and varies from 10 to 20 min.

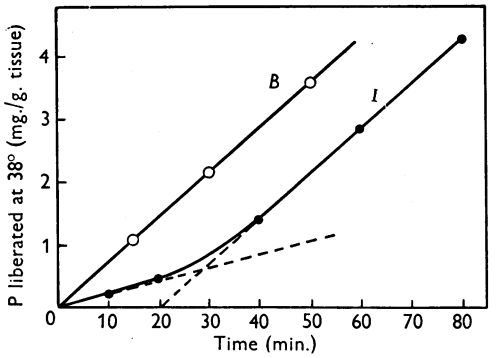

Fig. 3. Existence of lag period in activation at 38°. *I*, Washed mitochondria, intact; *B*, same preparation, treated 3 min. in the Waring blender. Standard incubation at 38° and pH 5.

It occurs even when the subsequent rate of activation is very great, as in the experiment represented in Fig. 3, in which full activation was reached between 20 and 40 min. incubation, the fastest rate of activation encountered so far in experiments of this type.

These experiments indicate that the true initial activity of preparations containing bound enzyme may be measured by the standard assay, provided that the incubation time does not exceed 10 min. An alternative method is to incubate at 0° and pH 6·1, in 0·25 M-sucrose containing 0·05 M- glycerophosphate.

Significance of the measured activities. The free activity observed with suspensions of washed mitochondria usually represents approximately 15 % of their total activity. A little less than half this amount is recovered in the supernatant fluid when the mitochondria are sedimented and is therefore due to fully active enzyme, set free in the process of resuspending the mitochondria. As will be shown in the following paper the residual activity which remains attached to the granules (8–10 % of the total) is due to a small amount of enzyme retained by non-specific and non-inhibitory adsorption to the surface of the mitochondria, the native bound form described in this paper having very little if any enzyme activity.

Consequently, the free activity of mitochondria, when determined under conditions which do not cause activation, is due essentially to the active enzyme present in the preparation, whereas the difference between the total and free activities must be considered a true measure of the amount of bound enzyme.

The quantitative distribution of acid phosphatase in the liver. In Table 6 are the results of a complete fractionation performed under what may be termed optimal conditions as the situation stands.

The technique described above was used, except for the following alterations. In order to minimize mechanical destruction of the mitochondria, these were washed only once; the supernatants from the centrifugations of the mitochondria were decanted very completely and subjected to an additional run of 10 min. at 8500 *g*, furnishing a small precipitate labelled 'intermediary fraction', and were then used for the separation of the microsomes.

Table 6. *The quantitative cytological distribution of acid phosphatase*

	Acid phosphatase activity					
	Fresh preparations (free enzyme)		Frozen preparations (total enzyme)		Difference (bound enzyme)	
Fraction	P*	%	P*	%	P*	%
Initial homogenate	265	17·1	1550	100	1285	82·9
Nuclei, washed twice	31	2	95	6·1	64	4·1
Mitochondria, washed once	87	5·6	950	61·3	863	55·7
Intermediary fraction, unwashed	33	2·1	190	12·2	157	10·1
Microsomes, washed twice	45	2·9	150	9·7	105	6·8
Final supernatant	134	8·6	163	10·5	29	1·9
Recovery	330	21·2	1548	99·8	1218	78·6

* μg. P/g. original tissue/10 min. All percentages calculated on the total activity, 1550 μg. P/g. tissue/10 min.

All the fractions were assayed for free enzyme activity by 10 min. tests, as soon as the fractionation was completed, and for total activity after freezing and thawing fifteen times.

Table 6 gives the amounts of free, total and (by difference) bound enzyme found in each fraction. The results show that the intial homogenate contains 83 % of bound enzyme and 17 % free. The distribution is slightly different when the data for the individual fractions is totalled, only 79 % of the enzyme being bound and 21 % free. These figures are entirely consistent with the observed damaging effects of the homogenizing and fractionation procedures, and strongly suggest that the whole of the acid phosphatase of liver is present in the intact cell in a mitochondria-linked form.

Of the complexes remaining intact 72 %, amounting to 56 % of the total activity, are recovered in the mitochondria fraction. A certain proportion is also found in other fractions, particularly in the intermediary and microsome fractions, which, in the Schneider procedure, would be combined into a single one.

As would be expected, a large part of the free activity is found in the final supernatant. The presence of free phosphatase in the other fractions is explained, partly by their content of fragile complexes, some of which are dissociated when the particles are resuspended, partly by their ability to adsorb the soluble enzyme.

Although only one complete experiment of this type has been performed, the distribution observed, in particular, the figure of 55–60 % for the recovery of acid phosphatase in the mitochondria, is fairly representative of the average of a large number of similar experiments. Thus in seven different homogenates of rat-liver tissue the mean total acid phosphatase activity, measured after exposure to the Waring blender or repeated freezing and thawing was $1{\cdot}52 \pm 0{\cdot}09$ mg. P/g. tissue/10 min., while the mean activity of sixteen preparations of mitochondria, in which somewhat less care was taken than in the foregoing experiment to recover the particles quantitatively, was $0{\cdot}82 \pm 0{\cdot}04$ mg. P/g. tissue/10 min., or 54 % of the mean total value.

A few qualitative experiments suggest that acid phosphatase is also mitochondria linked in the liver of rabbits and guinea pigs.

DISCUSSION

The results described above make it clear that a large part of the unspecific acid phosphatase of liver is linked to fairly large cytoplasmic granules in such a manner that the enzyme is inactive towards added glycerophosphate at pH 5. In all probability, the whole of the enzyme is particle bound in the intact cell.

The main questions raised by these findings are the nature of the linkage between enzyme and granule, the reason for the lack of activity of the bound enzyme and the nature of the phosphatase bearing granules. The two first questions will be dealt with more fully in the following paper. The third deserves some attention. From the general distribution of the bound enzyme, it would seem that the enzyme bearing particles belong to Claude's 'large granules', which have now been identified by numerous workers with the cell mitochondria. It should, however, be noted that a fairly large fraction of bound phosphatase fails to be recovered in the mitochondria fraction, but comes down with the microsomes and is even found to a small extent in the final supernatant. This fact, which comes out clearly in the results of Table 6, has been verified a number of times.

There are several possible interpretations for this observation, the simplest one being that a certain amount of sedimented mitochondria has been brought back into suspension, while decanting the supernatant after centrifugation. Although the possibility of some contamination occurring in this manner cannot be eliminated with certainty, it is doubtful whether it is the main factor responsible for the results obtained. In the first place, great care was taken in decanting the supernatant after centrifugation of the mitochondria and there was no indication that the packed precipitate had been disturbed in the course of this process. Neither did the supernatants contain any significant amount of granules visible at low magnifications. In addition, even after the supernatant has been subjected to an additional run of 10 min. at 8500 *g*, there is still found in suspension a significant amount (7 % of the total) of bound enzyme, which only sediments at 20,000 *g*, with the microsomes (Table 6).

Discounting the rather improbable possibility that mitochondria can be broken up into smaller fragments, while still retaining acid phosphatase in an inactive form, one is led to the conclusion that a given population of mitochondria may contain a significant proportion of smaller granules, which do not completely sediment in 10 min. at 8500 *g*. This, in turn, would appear to support the contention of Chantrenne (1947), who claimed on the basis of adenosinetriphosphatase and alkaline phosphatase measurements, that the distinction between mitochondria and microsomes is artificial and that a continuous spectrum of cytoplasmic granules exists, representing presumably various stages in the life cycle of these particles.

It is of particular interest in this connexion that liver tissue contains a second easily detectable enzyme, namely the specific glucose-6-phosphatase, which can be recovered to a very large extent in the microsome fraction, and which appears, from all the

evidence available, to be exclusively attached to submicroscopic granules (Hers *et al.* 1951). The distribution of this enzyme cannot be studied quite as accurately as that of acid phosphatase, owing to its lability and to the existence of interactions between the various fractions. However, the results, especially when they are corrected for the inactivation of the enzyme, which occurs mostly during the long centrifugations of the microsomes, provide a fair estimate of the distribution of glucose-6-phosphatase.

These measurements have been made on the fractions whose acid phosphatase activity is reported in Table 6 (Exp. 3 of Hers *et al.* 1951). It is therefore possible to establish in this particular experiment the content of each fraction of granules bearing acid phosphatase and glucose-6-phosphatase respectively. The distribution found is recorded in Table 7.

Table 7. *Distribution of acid-phosphatase- and glucose-6-phosphatase-bearing granules in fractions separated by centrifugation*

Fraction	Acid phosphatase-bearing granules (%)		Glucose-6-phosphatase-bearing granules* (%)	
Nuclei	4		6	
Mitochondria	56		13	
Intermediary	10	17	10	75
Microsomes	7		65	
Final supernatant	2		6	
Damaged	21		—	

* Corrected for inactivation of the enzyme.

The data of Table 7 make it clear that the distinction between microsomes and mitochondria is a valid one, but they also help to evaluate the limitations of the method of centrifugal fractionation in separating the two populations of granules. These limitations are not as apparent in other investigations in which the identification of the particles recovered in each fraction has rested mainly on chemical analyses and microscopic observations, and in which the distribution of more complex or less sharply characterized enzymic activities has been studied. In our experience the principal causes of error in a complete fractionation are the following.

Agglutination of particles with each other, and with particles of large size, causes them to come down in lower gravitational fields. As noted by other workers (Hogeboom *et al.* 1948; Kennedy & Lehninger, 1949; Hogeboom & Schneider, 1950), this phenomenon is enhanced by acidification, by an increase in ionic strength, and tends to increase with time. For instance, if the procedure for separating microsomes is shortened by centrifuging down the nuclei and the mitochondria simultaneously, as much as 95% of glucose-6-phosphatase can be recovered in the microsome fraction and in the final supernatant (Hers *et al.* 1951).

The relatively great variability in size of the particles in each population renders a certain amount of overlapping of the fractions inevitable. This appears to be true both for the microsomes and the mitochondria. In the former case, it may be estimated from glucose-6-phosphatase measurements that approximately 10% of the microsomes are too small to sediment completely in one hour at 20,000 *g* (Hers *et al.* 1951). The evidence presented here appears to show that an even larger proportion of the mitochondria is lost through incomplete sedimentation. It is our feeling that a better yield would be obtained by using a slightly higher gravitational field, such as 12,000–15,000 *g*, for the sedimentation of the mitochondria, for we have found that if only the upper half of the supernatant is collected after centrifuging 10 min. at 8500 *g*, practically no acid phosphatase bearing granules are recovered, an indication that even the lighter granules must already be well concentrated in the lower part of the tube at this stage.

The mitochondria are sensitive to mechanical damage. As will be shown in the following paper, the release of acid phosphatase is most probably the result of injuries to the mitochondrial membrane. The results presented in this paper show that such injuries are very easily sustained and that as much as 20% of the mitochondria may be damaged in this manner in the course of a fractionation experiment.

It is of course not possible to decide whether these conclusions are applicable to all cytoplasmic granules, since the general denomination of mitochondria or microsomes may cover a large number of populations of particles, similar in size and sedimentation rate, but differing in many other properties. That their applicability may not be restricted exclusively to the granules studied here is suggested by the fact that approximately 50% of another soluble protein which appears to be loosely bound to the mitochondria, namely cytochrome *c*, has been recovered, in the mitochondria fraction by Schneider & Hogeboom (1950). Indeed, the authors consider the possibility that all the cytochrome *c* may be mitochondria bound in the intact liver cell, a hypothesis which would appear to gain in probability from the results described in the present paper.

A study of the intracellular distribution of acid phosphatase in rat-liver cells has just been published by Palade (1951). The distribution found by this author is fairly similar to that described in this paper, except that he finds relatively less enzyme in the mitochondria fraction (40%), and relatively more in the other fractions. This difference can probably be explained by the fact that Palade's homogenates are prepared in hypertonic sucrose

(0·88 M) and are fractionated according to a scheme which is not directly comparable with ours. It is significant that in his experiments also, a fairly large proportion of the enzyme fails to sediment in gravitational fields sufficient to bring down all the large granules, and is partly recovered with the smaller granules.

A more important difference between Palade's work and ours lies in the conditions used for the enzyme assays. Palade's tests are performed in acetate buffer pH 4·5, with 0·08 M-β-glycerophosphate as substrate. Enzyme and substrate are first equilibrated separately for 10 min. at 38° in the two parts of a Warburg vessel, mixed and incubated for 1 hr. with continuous shaking. These conditions favour activation during the assay and probably explain why Palade did not observe the low activity of the bound enzyme or the various types of activation described in this paper.

As pointed out above, these features have an important bearing on the final interpretation of the results of distribution experiments.

SUMMARY

1. In fresh rat-liver homogenates prepared in 0·25 M-sucrose, a large proportion of the acid phosphatase is linked with the mitochondria.

2. The bound enzyme has little or no activity towards added glycerophosphate at pH 5.

3. Ageing of the preparations at 0°, short exposure to the Waring blender, repeated freezing and thawing, all lead to an irreversible release of the enzyme, which then appears to a large extent in a soluble form. Partial damage to the complex is caused by the procedures used to homogenize and fractionate liver tissue.

4. In contrast to its bound form, the free enzyme exhibits a high activity.

5. When mitochondria are separated by differential centrifugation and washed once, they are found to contain 55–60 % of the total acid phosphatase content of the original homogenate. The remainder is found partly in the smaller granules, partly in the final supernatant. The nuclear fraction contains only a low percentage of the total activity.

6. Analysis of these results indicates: (*a*) that the whole acid phosphatase content of rat liver must be particle-bound in the intact tissue; (*b*) that the ability to bind acid phosphatase in the manner described belongs to a population of granules which are fairly heterogeneous in size. About one-third of them are too small to sediment completely in 10 min. at 8500 *g*.

7. The implications of these findings, in particular with respect to the technique of centrifugal fractionation, are discussed.

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